Introduction of the α Subunit Mutation Associated with the B1 Variant of Tay-Sachs Disease into the β Subunit Produces a β-Hexosaminidase B without Catalytic Activity*

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β-Hexosaminidase is a lysosomal glycosidase. In normal human tissues it occurs in two isozyme forms, A and B. Hexosaminidase A is a heterodimer composed of one α and one β subunit, while hexosaminidase B is a homodimer of two β subunits. The prepro-α and β chains are coded for by the HEXA gene (chromosome 15) and HEXB gene (chromosome 5), respectively. In the past several years, cDNA clones coding for the α and β subunits have been isolated by ourselves (1, 2) and others (3, 4). The comparison of the deduced primary sequences of the α and β subunits showed an overall 60% homology, suggesting a common evolutionary origin (2). This was confirmed when the HEXA and HEXB gene structures were determined. Of the 14 intron-exon junctions present in each gene, 13 were mapped to identical sites on the aligned cDNA sequences (5). Thus, the functional domains in each subunit might well be structurally and spatially related.

The isolation of the cDNAs has also allowed the elucidation of much of the proteolytic (6-10) and oligosaccharide side chain (11, 12) processing, leading to the complex subunit structures present in the mature, lysosomal isozymes. Proteolytic processing converts the single pro-α chain into a mature α subunit composed of two chains linked by a disulfide bond, a major 53-kDa polypeptide (13, 14), and ε recently described minor 7-kDa peptide (10). Similarly, the mature β subunit contains three chains also linked by disulfide bridges, two major chains, βε (30 kDa) and βδ (24-26 kDa) (15), and a minor peptide of 7-10 kDa (9, 10, 12). The generation of the mature 24-30 kDa β chains from the single 65-kDa pro-β chain, easily detectable by SDS-PAGE,1 has long been used as an indicator of incorporation into the lysosome (14, 16).

While both hexosaminidase A and B are able to hydrolyze many of the same natural and artificial substrates containing terminal nonreducing β-linked GlcNAc or GalNAc residues, only hexosaminidase A can utilize substrates containing a GlcNAc-6-sulfate residue, e.g., MUGS and certain glycosaminoglycans, or in combination with a specific activator protein, can hydrolyze GalNAc from Gα5 ganglioside. Indeed, these observations, when coupled with the finding that hexosaminidase B (in the presence of detergent to substitute for the activator protein) can hydrolyze Gα5, the asialo derivative of Gm5, led Kresse et al. (17) to postulate that the active sites of the two subunits differ by the presence of a positively charged substrate-binding pocket in the α subunit (for the sialic acid or sulfate group) that is either inaccessible or negatively charged on human gangliosides, e.g. GM2 ganglioside. Recently, a point mutation, producing a single amino acid substitution in the α subunit (Arg17'-His), has been found to be associated with the B1 variant phenotype of Tay-Sachs disease (Ohno, K., and Suzuki, K. (1988) J. Neurochem. 50, 316-318). This variant is characterized by normal levels of hexosaminidase A as measured by a common artificial substrate, but an absence of activity toward α subunit-specific substrates. However, because of the presence of an active β subunit in the mutant hexosaminidase A, it has not been possible to determine whether the affected α subunit has undergone a change in substrate specificity or become totally inactive. In order to define the full effect of the B1 mutation we have taken advantage of the common evolutionary origin of the genes coding for the α and β subunits. Since the B1 mutation occurs in a region of extended identity between the two subunits, we have duplicated the Arg17'-His mutation in a cDNA coding for the human β subunit (Arg17'-His). By expression of the mutant construct in monkey COS cells we have been able to examine the effect of this mutation on β subunits which are capable of forming stable, active homodimers, an experiment that could not readily be accomplished with heterodimeric hexosaminidase A. Our data show that β homodimers containing the Arg17'-His substitution are formed and are transported into the lysosome in a manner identical to that of normal pro-hexosaminidase B. However, the mutant homodimers are processed at a slower rate and are less stable in the lysosome. Their most striking feature was a total lack of normal hexosaminidase B activity. We conclude that while the effect of the Arg17'-His substitution is not strictly limited to the active site, the severe B1 phenotype results from a totally inactive α-subunit in hexosaminidase A.

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1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gm5, GalNAc(1-4)-[NANA(2-3)]-Galβ(1-4)-Glc-ceramide; MU, methylumbelliferyl-4-MUG, 4-methylumbelliferyl-β-N-acetylgalcosaminide; 4-MUGS, 4-methylumbelliferyl- β-N-acetylgalcosaminide-6-sulfate; HPLC, high performance liquid chromatography.
charged in the α subunit. This theory was strengthened by the observation that the active sites of the α and β subunits differ in their K\textsubscript{cat} values toward neutral, i.e. 4-MUG, and negatively charged artificial substrates, i.e. 4-MUGS (18).

Much of the interest in hexosaminidase A has come from investigations of inherited disorders known as the GM\textsubscript{2} gangliosidoses, a group of severe neurodegenerative diseases characterized by the lysosomal accumulation of GM\textsubscript{2} in brain and peripheral nervous tissue. One of these, Tay-Sachs disease, results from mutations of the HEX\textsubscript{A} gene. Here there is a deficiency of hexosaminidase A but not hexosaminidase B activity since only the α subunit is affected. In Sandhoff disease, caused by mutation of the HEX\textsubscript{B} gene, both enzyme activities are affected due to loss of the common β subunit. The rare, AV variant, is associated with deficiency of the GM\textsubscript{2}-activator protein (19–21).

An unusual variant of Tay-Sachs disease is known as the B1 variant. Originally thought to be related to the AB variant, patients affected with this disease express both hexosaminidase A and B activity as assayed with neutral (common) substrates, e.g. 4-MUG. However, unlike the normal hexosaminidase A found in the true AB variants, Kytizis et al. (22) found that B1 variant hexosaminidase A migrated toward a GlcNAc-6-sulfate containing substrate and suggested the presence of a mutation at or near the active site of the α subunit. A similar hypothesis was advanced by Suzuki and colleagues (23, 24) who identified a specific mutation linked to the B1 phenotype in five of six patients examined. The mutation is a nucleotide substitution, G to A, which changes Arg (178) to His in the α subunit. They noted that this site is found in a stretch of 8 identical amino acids in the α and β subunits (Fig. 1), and that this substitution is predicted (by computer analysis) to cause a change in the secondary structure of the α polypeptide. More recently 5 of these 8 identical residues, including the Arg, have been found to be conserved in species as diverse as Dictyostelium discoideum (25) (Fig. 1).

However, knowledge of the exact α mutation, even when coupled with the biochemical characterization of the hexosaminidase A from B1 patients, has not fully defined the impact of the Arg\textsuperscript{178}His substitution on the α subunit. This is because of the presence of a normal, active β subunit in the hexosaminidase A heterodimer. For example, it is not known whether the affected α subunit has undergone a partial change in substrate specificity, i.e. become β-like and can no longer bind negatively charged substrates but can still hydrolyze neutral substrates, or whether it has lost its catalytic ability entirely. Furthermore, subtle changes to the stability, capacity for intracellular transport, or processing of the α subunit could well be masked by the presence of the normal β subunit.

The B1 mutation provides a starting point for the identification and characterization of the protein domains that are involved in substrate binding and hydrolysis in the α and β subunits. In this study, we have examined the effect of the Arg\textsuperscript{178}His mutation by introducing it into the β subunit at the homologous position (Arg\textsuperscript{211}His). We show that expression of the "B1-like" β subunit in monkey COS cells produces a homodimeric B1-like hexosaminidase B. We examined the impact of this substitution on the synthesis, intracellular transport, and stability of the mutant band as well as its effects on the catalytic activity of a β homodimer, an experiment that could not be readily accomplished with the heterodimeric (αβ) A-isozyme.

MATERIALS AND METHODS

Site-directed Mutagenesis and Vector Constructions—General cloning procedures were as described by Maniatis (26). A 500-base pair EcoRI DNA fragment from pHexB43 (5) was subcloned into pBS+ (Stratagene). A 48-mer oligonucleotide, 5'-GGAATTTTGGATTTACATCCCACCATATTGTCAAG-3', was synthesized for the mutagenesis. It contains the change (underlined in sequence), AGA→CAC, to convert Arg\textsuperscript{211}His in the deduced amino acid sequence of the β subunit (2). The mutagenesis was performed according to published procedures (Amersham Corp.), based on the original method described by Taylor et al. (27). The mutation was verified by sequencing the entire 500-base pair fragment using the dyeoxy chain termination method (28). The mutant insert was subcloned back into pHexB43 for expression studies.

Cell Culture—Cos-1 monkey kidney cells (American Type Culture Collection) were maintained in α-minimum essential media (Flow Laboratories) containing glutamine, tryptophan and penicillin (100 units/ml), and 10% fetal bovine serum with or without the addition of 20 μM leupeptin at 37 °C in 5% CO\textsubscript{2}.

DNA Transfection—The pcD-derived plasmid, pHexB43 (constructed with the SV40 Ori and early promoter for expression in mammalian cells (29)) which contained the wild type or B1-like hexosaminidase B isozyme, was transfected into monkey COS-1 cells using the CaPO\textsubscript{3} procedure as previously described (30).

Solid-state Immunoprecipitation Assay for Hexosaminidase B—Cell lysates and media were analyzed for human hexosaminidase activity by a solid-state immunoprecipitation assay using 4-MUG as a substrate. The assay was similar in that linear is the optimal concentration of 4-MUG (data not shown). COS lysate, 0.25-2 μl of a total 50 μl (obtained from one confluent 100 x 20-mm tissue culture dish) in 70 μl of phosphate-buffered saline, or 500 μl of cell culture medium was rotated at 4 °C overnight with 15 μl of protein-A Sepharose beads (Pharmacia LKB Biotechnology Inc.) and 10 μl of polyclonal rabbit anti-human β-IgG prepared in our laboratory. After centrifugation for 15 min at 11,300 x g the supernatant was removed and analyzed for COS hexosaminidase (A and B) activity, while the beads were washed (3 x with phosphate-buffered saline) and assayed directly to determine total human hexosaminidase B activity by a MU fluorescence assay (31).

Western Blot—SDS-PAGE was used to analyze the remaining COS lysate. Equal amounts of total protein were examined according to the method of Laemmli (32) using a Bio-Rad Mini Gel system (1-mm thick slab gels with 10% polyacrylamide separating gel and 4.5% stacking gel). Proteins were transferred overnight to nitrocellulose. The Western blot was developed by the peroxidase antiperoxidase procedure (Jackson Immunological) using a donkey anti-rabbit IgG (heavy and light chains) as the bridging antibody and normal donkey serum as a blocking agent.

Molecular Sieve Chromatography—The COS cells (grown in media containing 20 μM leupeptin) from two 100 x 20-mm tissue culture dishes, transfected with either the wild type pHexB43 or the construct containing the B1-like mutation, were harvested. Each lysate was cleared with carbon tetrachloride and injected into a 15 x 500-mm HiLoad Superose 12 column (Superose 12 from Pharmacia) calibrated with a blue dextran (void volume), aldolase (105 kDa), purified placental hexosaminidase B (120 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and vitamin B\textsubscript{12} (total volume of the column). The column was eluted at 0.5 ml/min with a 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 0.1 mM dithiobitol. Protein was detected by monitoring the column effluent at 280 nm. Fractions, 0.5 ml, were collected at the column void volume, and at 1-min intervals from 22-30 min in order to span the Mr, region predicted for β dimers and monomers. Each 0.5-ml fraction was dialyzed against 4 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 0.02% SDS and 10 mM sodium phosphate buffer, pH 7.0, containing 0.02% SDS and 0.5 M NaCl and dialyzed against 30 μl of water containing 50 mM dithiobitol, 20% glycerol, and 0.02% bromphenol blue. Half of each sample was examined by SDS-PAGE followed by Western blot analysis.
RESULTS

Hexosaminidase Activity—Initially, hexosaminidase activity towards the conventional 4-MUG substrate was measured in human fibroblast and monkey COS cell lysates to establish linearity and to compare free and bound activity in the solid phase, immunoselective, enzyme assay (Table I). All raw assay results fell below the predetermined maximum for linearity and protein A-Sepharose anti-human β-IgG capacity, <70 nmol/h (data not shown). Activities were normalized by dividing by the total protein in the cell lysates, 0.6–21 μg, added to each binding assay to give a specific activity (Table I). The solid-state assay bound about 90% of the hexosaminidase activity from normal human fibroblasts (Table I; fibroblast(+), (free + bound)/bound) with a yield of about 80% of the original lysate value. In contrast, the activity bound from mock-transfected COS cells was only 4% of the total (Table I, COS(−)). This experiment showed that the protein A-Sepharose-bound β antibody raised against the human subunit recognizes the human enzyme in preference to the COS-enzyme monkey. This was further substantiated by Western blotting, shown below.

COS cells were transfected with pHexB43, an expression plasmid containing the full-length β-cDNA driven by the SV40 early promoter, and incubated for 48 h to allow expression of the enzyme. The specific activity of the lysate towards 4-MUG was increased by 13-fold over the endogenous COS activity (Table I, compare lysate values for COS(+) and COS(−)). However, because of the specificity of the solid-state assay for the human enzyme (above) a 300-fold increase was found in the specific activity that could be immobilized on the protein A-anti-human IgG beads (Table I; bound COS(+) compared to bound COS(−)). Similar results were found by utilizing the solid-state assay to determine the enzyme activity secreted into the culture medium (data not shown). Thus, the pHexB43 plasmid directs the synthesis of an active hexosaminidase that, like the authentic human fibroblast enzyme, is preferentially bound by the anti-human β-IgG. Furthermore, the use of the immunoselective assay allows for the detection (>double the negative control) of <1% of the activity produced by the wild type construct.

We next examined the amount of hexosaminidase 4-MUG activity produced by three independent transfections utilizing the pHexB43-based construct containing the B1-like Arg311 mutation. In each experiment, the specific activity of the immobilized enzyme was very low (<double the negative control), about 200-fold less than was immobilized in the positive control (Table I; B1, 1–3). Human hexosaminidase activity measurements of the cell culture media produced similar results (data not shown). These results demonstrated that no significant amount of human hexosaminidase B activity is produced in COS cells transfected with the mutant cDNA plasmid.

Western Blotting—In order to confirm that the plasmid containing the mutant insert indeed directed the synthesis of a protein, and to determine the extent to which the mutant protein was processed and delivered to the lysosome, the hexosaminidase produced by transfected COS cells was examined directly by Western blotting. This would allow us to assess whether the deficient activity described above was due to a decrease in the stability of the protein, or an inability to hydrolyze the 4-MUG substrate.

Initial experiments showed that the pattern of immunoreactive protein bands produced by COS cells transfected with pHexB43 was identical to that seen in human fibroblasts with the pro-β precursor band at a position corresponding to a M, of 65,000 and the mature β band at 30 kDa. The immunoreactivity of the smaller human β, chains appeared to be less and could only be visualized with an increased protein load or extended development time. Endogenous COS hexosaminidase polypeptides were not seen; however, when samples were considerably overloaded, faint bands corresponding to the COS monkey hexosaminidase pro-β and β, chains (in positions identical to their human components) could be seen (data not shown).

Western blots of COS cell extracts from the three independent transfections with the B1 mutant cDNA described in Table I were compared to that of an extract from cells transfected with the wild type construct. In all these cases, a steady-state presence of approximately 20% of fully processed β chains was evident in each of the COS B1 extracts (data not shown; see patterns of identical transfections in Fig. 2, −leupeptin lanes). Thus, the mutation has either produced a protein that is less stable, once in the lysosome, or is defective in intracellular transport, i.e. most of the mutant β-

![Fig. 2. Western blots of equal amounts of protein from COS cell lysate. Cells were grown without (−leupeptin) or with 20 μM leupeptin (+leupeptin) after transfection with the normal pHexB43 plasmid, COS(+), or the mutant pHexB43 plasmid coding for the Arg311-His substitution, B1. Mock-transfected COS cells were used as the negative control, COS(−). Prestained protein M, standards can be seen at the right with relevant M, indicated. The positions of immunoreactive bands corresponding to the pro-β polypeptide and mature β, chain of hexosaminidase are shown on the left.](image-url)
protein is trapped in the endoplasmic reticulum and is degraded, as had been shown to occur for other mutant proteins (39), or it has been passed along the secretory pathway.

**Analysis of the Intracellular Hexosaminidase in Transfected COS Cells Grown with Leupeptin—** To determine if the mutation had affected the stability of the protein in the lysosome, COS cells were transfected with wild type or mutant pHexB43 and grown in media containing 20 μM leupeptin, a tripeptide thiol and serine protease inhibitor that has been used to increase the stability of proteins in the lysosome (34). The human β polypeptides from transfected cells grown with or without leupeptin were examined by Western blotting. While there was a noticeable increase in the intensity of the bands, marked by the lack of the bands in the leupeptin lanes. However, both experiments demonstrated a 2-fold increase in the bound hexosaminidase-specific activity when the positive control (COS(+)) was grown in leupeptin (Fig. 2). The mature β chains in the B1 sample were present in both the COS(+) and the B1 sample. Since the β and β chains are produced in the lysosome (14, 16), these data show that the mutation affects the stability of the protein in the lysosome, rather than intracellular transport.

Specific activity measurements from two separate experiments are presented in Table II. The absolute values varied between experiments. This variation was presumably due to a change in transfection efficiency with the use of new reagents (there is a very narrow pH optimum for transfection with CaPO4 (35)). However, both experiments demonstrated a 2-fold increase in the bound hexosaminidase-specific activity when the positive control (COS(+)) was grown in leupeptin. Significantly, the large increase in the amount of COS B1 protein seen in Fig. 2 was not reflected in a proportional increase (over the negative control) in the amount of residual 4-MUG activity bound in the solid-state assay. Thus, we conclude that the COS B1 extracts are essentially devoid of activity towards the neutral (common) substrates.

**Molecular Weight Determinations of the Translation Products—** Since dimer formation is necessary to confer enzyme activity, the native M, of the hexosaminidase B-derived from B1-like and wild type pHexB43-transfected COS cell extracts was examined by HPLC sieve chromatography (Fig. 3). Fractions were collected which include the void volume of the column, 14–16 min (to detect aggregates which are also inactive (13)), and fractions at 1-min intervals spanning the elution ranges of the β-dimers and monomers M, (22–30 min). The mature β chains in each fraction were detected by Western blotting (Fig. 3). When the intensities of the immunoreactive bands in each fraction from the positive control were compared, the peak in banding intensity corresponded to the fraction taken at between 24 and 25 min. This area was the same in which purified placental hexosaminidase B eluted. No immunoreactive bands were observed in the column void volume (Fig. 3, COS(+)). The same results were obtained when the fractions containing the B1-like β protein were examined (Fig. 3, COS B1). Thus, no β monomers or aggregates were present in either the COS(+) or the B1 sample and human hexosaminidase B composed of two β subunits, each containing the Arg→His mutation, was formed.

**DISCUSSION**

Several explanations can be advanced to explain the inactivation of hexosaminidase B as a result of introducing the

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**Table II**

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Sample</th>
<th>Specific activity, † leupeptin</th>
<th>nmol/h/μg</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>COS(+)</td>
<td>12.3</td>
<td>29.4</td>
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<tr>
<td></td>
<td>COS(−)</td>
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<td>0.10</td>
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<tr>
<td></td>
<td>COS B1</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>COS(+)</td>
<td>4.8</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>COS(−)</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>COS B1</td>
<td>0.12</td>
<td>0.07</td>
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*Separate experiments, each using newly prepared reagents, displayed variations in transfection efficiencies, presumably due to the narrow pH optimum for DNA uptake by cells using the CaPO4 procedure (35).
† The nanomoles of 4-MU produced by the human β-hexosaminidase B bound by protein A Sepharose anti-human β-IgG/μg of soluble COS cell lysate protein, as calculated in Table I.
* Extract from COS cells transfected with normal pHexB43, positive control.
† Mock-transfected COS cell extract, negative control.
* COS cells transfected with the mutant pHexB43 coding for the Arg→His substitution.
Arg211-His mutation into the β subunit (Table I). Such an outcome could result from; 1) protein instability either in the lysosome or in the endoplasmic reticulum (due to the failure of the mutant protein to properly fold and exit), 2) failure of the protein to be properly compartmentalized resulting in secretion, 3) an inability to form dimers, or 4) a local change affecting the active site of the isozyme. The issue of stability was addressed by examining the enzyme by Western blotting. When compared to the positive control there was only about 20% as much mature (24–30 kDa) lysosomal mutant β chains present intracellularly. There was also a noticeable increase in the pro-β/β ratio for Arg211-His substituted β polypeptides (Fig. 2, −leupeptin(−) lanes, compare COS(+) to B1). Thus, while the activity measurements indicate that the residual intracellular 20% of the B1-like protein was indeed inactivated by the mutation (through an effect on the active site or through a lack of dimer formation), the mutation must also affect either protein stability or intracellular transport.

In order to determine if stability of the protein in the lysosome was affected, COS cells were transfected and grown in the presence of 20 μM leupeptin. The amount of mature B1-like β chains was increased by this procedure to levels comparable to the positive control (Fig. 2, (+)leupeptin lanes), indicating that the B1 mutation affects the intralysosomal stability rather than transport of the B1-like β chains. It was also noted that the increased pro-β/mature β ratio in the B1-like mutant was retained with growth in the presence of leupeptin. This finding suggests that the rate of pro-β chain maturation in the lysosome is also affected by the B1 mutation. If the transport of β precursor, rather than lysosomal maturation, had been slowed, the amount of mature (as βm) β chains, but not pro-β chains would have increased. We conclude, therefore, that the introduction of the B1-Arg211-His mutation into the β subunit of hexosaminidase decreases the stability of the mutant protein in the lysosome, and the rate of lysosomal processing of the resulting pro-protein.

With levels of the intracellular B1-like protein increased to control levels by growth in leupeptin, we re-evaluated the amount of 4-MUG activity in the lysate of these cells (Table II). In two separate experiments we found that there was no significant increase in activity, i.e. <double the negative control values. Thus, the B1 mutation produces a lysosomal β protein that is totally devoid of normal hexosaminidase B activity. It remained for us to determine whether this was caused by a local effect on the active site or a more general failure in dimer formation.

The approximate native Mr of the B1-like β protein from lysates of COS cells grown in leupeptin was determined by HPLC sieve chromatography. When compared to the elution profiles of purified placental hexosaminidase B and the positive control COS cell lysate, there was no difference in the point of maximal elution from the sieve column (Fig. 3). Therefore, dimer formation of the β subunits containing the Arg211-His substitution is uninhibited.

Previous comparisons of the deduced primary structures of the α and β subunits and the intron-exon junctions of the HEXA and HEXB genes (2, 3) demonstrated their common evolutionary origin. From these findings it would be expected that functional domains within the α and β subunits are conserved. The data presented in this report, demonstrating that the Arg211-His substitution in the β subunit produces a hexosaminidase B with biochemical characteristics consistent with those measurable for the hexosaminidase A from B1 patients (containing an Arg178-His substituted in the α subunit), provide direct experimental evidence of such a conservation. Furthermore, the ability to examine stable Arg211-His substituted β homodimers demonstrated that the naturally occurring B1 phenotype related to a totally inactive α subunit in the affected β dimmer, hexosaminidase A, rather than one that has lost its ability to hydrolyze only negatively charged substrates. The decrease in lysosomal stability of the mutant hexosaminidase B and the possible slower rate of lysosomal processing does indicate that a more general change in protein structure has also occurred, as previously predicted by computer analysis (23). These observations leave open the question of whether the βArg211 and αArg178 residues are actually a part of the substrate binding/catalytic sites. However, because of the necessarily limited extent of this structural change, reflected in the ability of the protein to form dimers and be successfully transported into the lysosome, the residue must at least be in close proximity to such a critical site. Further work is needed to determine the effect of more conservative changes in and around the region identified through the characterization of this naturally occurring mutation.

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REFERENCES

Introduction of Mutation into the β Subunit of β-Hexosaminidase


